



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

### **Specific selection of antigen-reactive lymphocytes into antigenically stimulated lymph nodes in sheep**

**Citation for published version:**

Hopkins, J, McConnell, I & Lachmann, PJ 1981, 'Specific selection of antigen-reactive lymphocytes into antigenically stimulated lymph nodes in sheep', *Journal of Experimental Medicine*, vol. 153, no. 3, pp. 706-19.

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

Journal of Experimental Medicine

**Publisher Rights Statement:**

© 1981 The Rockefeller University Press

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



# SPECIFIC SELECTION OF ANTIGEN-REACTIVE LYMPHOCYTES INTO ANTIGENICALLY STIMULATED LYMPH NODES IN SHEEP\*

By J. HOPKINS, I. McCONNELL, AND P. J. LACHMANN

*From the Mechanisms in Tumour Immunity Unit, MRC Centre, Cambridge, CB2 2QH, England*

Antigen-specific trapping of recirculating lymphocytes in vivo increases the number of immunocompetent cells available for the induction of the immune response. This recruitment of specific cells into lymphoid tissue also results in their selective depletion from the recirculating lymphocyte pool (RLP)<sup>1</sup> (1).

Experimental evidence for this is shown by the fact that shortly after intravenous challenge of mice or rats with heterologous erythrocytes (2-4) or allogeneic cells (5, 6), lymphocytes specific for these antigens become depleted from the thoracic duct lymph (TDL). This depletion is matched by a net increase in the number of specific cells in the spleen, the main site of antigen localization (7). Similarly, if parental-strain lymphocytes are adoptively transferred to irradiated F<sub>1</sub> recipients, then the parental cells appearing in the TDL of the F<sub>1</sub> recipient 36 h later have little reactivity to recipient alloantigens but normal reactivity to third-party alloantigens (7). Finally, antigen present in lymph nodes has also been shown to be able to produce the specific accumulation of antigen-reactive cells in this tissue (8, 9).

It is generally assumed that the mechanism of specific trapping of cells by antigen is the same in spleen and lymph nodes. That is, antigen localized to the surface of antigen-presenting cells within lymphoid tissue gradually abstracts all the specific cells as a result of repeated and random passage of the RLP through each lymphoid organ (1, 10). This seems a plausible explanation for the trapping of specific cells in the spleen where the total RLP passes through the organ in the blood in a very short time. However for lymph nodes the situation may not be entirely identical because only a fraction of the RLP present in the blood randomly enters a single node at any one time.

There are two sets of observations that show that antigen-specific localization of cells within lymph nodes can occur even when the efferent lymphatic from the node is cannulated (11, 12). In this situation, repeated passage of the specific cells cannot occur because all cells that enter the node are lost from the RLP via the indwelling cannula. In our own study (11) it was shown that if the efferent lymphatic from a single lymph node in sheep was cannulated and the node repeatedly challenged with

\* Supported by the Cancer Research Campaign.

<sup>1</sup> *Abbreviations used in this paper:* ABC, antigen-binding capacity; CG, chicken globulin; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; MLC, mixed lymphocyte culture; NIP, 4-hydroxyl-5-iodo-3 nitrophenyl acetyl; NIP-CAP, 4-hydroxyl-5-[<sup>125</sup>I]iodo-3-nitrophenyl acetyl caproic acid; PBL, peripheral blood lymphocytes; PCV, post-capillary venule; PPD, purified protein derivative of tuberculin; RLP, recirculating lymphocyte pool; SRBC, sheep erythrocytes; TDL, thoracic duct lymph; TNP, trinitrophenyl sulphonc acid.

antigen, then the entire animal became specifically unresponsive to the challenge antigen. Because the cells and antigen present within the node are lost from the animal via the cannulated lymphatic, other explanations based on tolerance induction or active suppression seem unlikely. Preliminary observations by Cahill et al. (12) similarly showed that challenge of a cannulated popliteal node with allogeneic cells resulted in the loss of alloreactive cells from a nonstimulated, contralateral node.

This paper confirms our earlier observations. In addition, it shows that there is a gradual and specific loss of antigen-reactive cells from the RLP in the blood after repeated challenge of a cannulated node with antigen.

### Materials and Methods

**Animals and Surgery.** 1- to 2-yr-old Finnish Landrace sheep were obtained from our own breeding flock. They were kept in standard pattern metabolism cages and were fed on hay with water and salt lick *ad libitum*. The efferent popliteal lymphatic vessels were cannulated as described (13). Animals were allowed 48 h to recover before the first injection of antigen. During the experiment, lymph samples were collected quantitatively into sterile, heparinized bottles.

**Antigens.** Purified protein derivative of tuberculin; batch 297, (PPD), and johnin (a similar purified protein derivative from *Mycobacterium paratuberculosis*; batch 298) were obtained from the Central Veterinary Laboratories, Weybridge, Surrey. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif. They were made to 50  $\mu\text{g}/\text{ml}$  in the culture medium, sterilized by filtration (0.22  $\mu\text{g}$  Millex) and stored at  $-20^\circ\text{C}$ . The hapten 4-hydroxyl-5-iodo-3-nitrophenyl acetyl (NIP) was synthesized by Dr. M. J. Hobart using the method of Brownstone et al. (14) and conjugated to PPD and chicken globulin (CG) by the method of Brownstone et al. (15). Trinitrophenyl sulphonic acid (TNP; BDH Chemicals Ltd., Poole, Dorset, England) was conjugated to johnin, KLH, and autologous sheep erythrocytes (SRBC) by the method of Rittenberg and Amkraut (16) and Rittenberg and Pratt (17). Allogeneic peripheral blood lymphocytes (PBL) were obtained from a 2-yr-old Clun Forest sheep and were prepared from the jugular blood by standard methods using Ficoll-Hypaque separation.

**Culture Medium.** RPMI-1640 buffered by  $\text{NaHCO}_3$  at 2.2 g/liter with 10% FCS (both from Gibco-Biocult Ltd, Irvine, Scotland) and supplemented with antibiotics, fungizone (E. R. Squibb and Sons, New York), and 2-mercaptoethanol at  $2 \times 10^{-5}$  M.

**Preparation of PBL.** Blood was taken by venepuncture and defibrinated using glass beads. The defibrinated blood was diluted two times with Hanks' balanced salt solution (HBSS) and the lymphocytes isolated by centrifugation over Ficoll-Hypaque (18). Cells were washed twice and made to a concentration of  $1.25 \times 10^6/\text{ml}$  in the culture medium.

**In Vitro Transformation of PBL.** Transformation assays were performed in sterile, round-bottomed microtiter trays (Sterilin Ltd, Teddington, Surrey, England). Four concentrations of cells were used:  $1.25 \times 10^5$  (100  $\mu\text{l}$  of cell suspension/well);  $0.625 \times 10^5$  (50  $\mu\text{l}$ );  $0.25 \times 10^5$  (20  $\mu\text{l}$ ) and  $0.125 \times 10^5$  (10  $\mu\text{l}$ ). Allogeneic stimulator cells for mixed lymphocyte cultures (MLC) were washed twice in HBSS and made to a concentration of  $1.5 \times 10^6/\text{ml}$  in the culture medium. They were then incubated with mitomycin C (Sigma Chemical) at 25  $\mu\text{g}/\text{ml}$  for 30 min at  $37^\circ\text{C}$ . After washing these cells three times, they were made to a concentration of  $1.25 \times 10^6/\text{ml}$  in the culture medium.

100  $\mu\text{l}$  (5  $\mu\text{g}$ ) of antigen (either PPD, johnin, or KLH) or 100  $\mu\text{l}$  ( $1.25 \times 10^5$ ) mitomycin C-treated allogeneic PBL were then added (five replicates of each). The final vol of all cultures was made to a concentration of 200  $\mu\text{l}$ . The plates were incubated for 5 d at  $37^\circ\text{C}$  in 7%  $\text{CO}_2$ . Control cultures were set up without antigen. 10  $\mu\text{l}$  (1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (Radiochemical Centre, Amersham, England) was added to each well for the final 24 h incubation. The cultures were harvested onto Whatman GF/A filter (Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England) mats using a Titertek microtitration harvester (Flow Laboratories, Inc., Rockville, Md.). The radioactivity of each sample was measured by  $\beta$ -spectroscopy using toluene-2,5-diphenyloxazole (PPO) as scintillant. The results are expressed as  $\log_{10}$  of the square

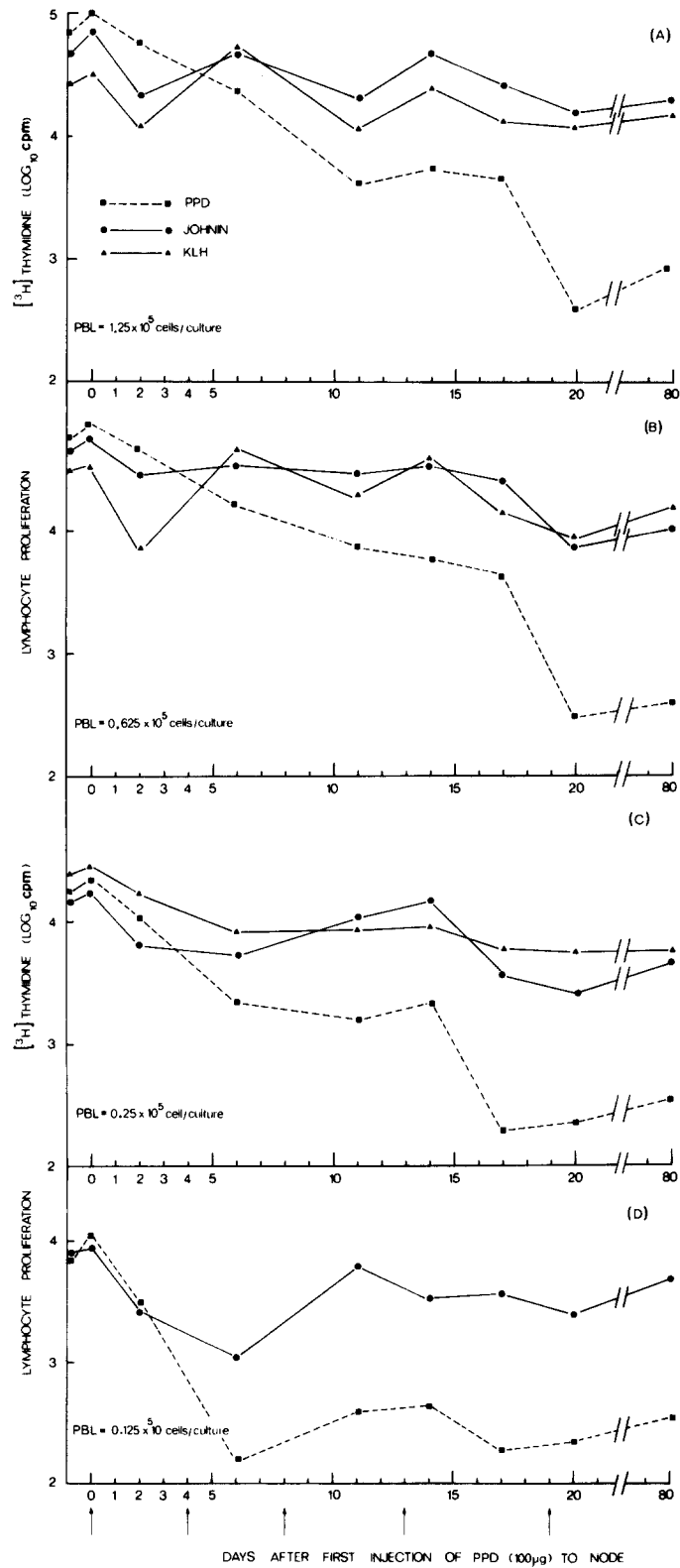


FIG. 1. In vitro transformation (by PPD, johnin, or KLH) of PBL of sheep DF28 that had been given five injections of 100 µg PPD into the drainage area of a cannulated popliteal lymph node (↑). The four sections (a-d) show results from four different cell concentrations. In all cases the results are expressed as log<sub>10</sub> cpm [<sup>3</sup>H]thymidine incorporation. The standard deviation within the replicates (5 times) of individual assays was always < ± 15% of the root square mean.

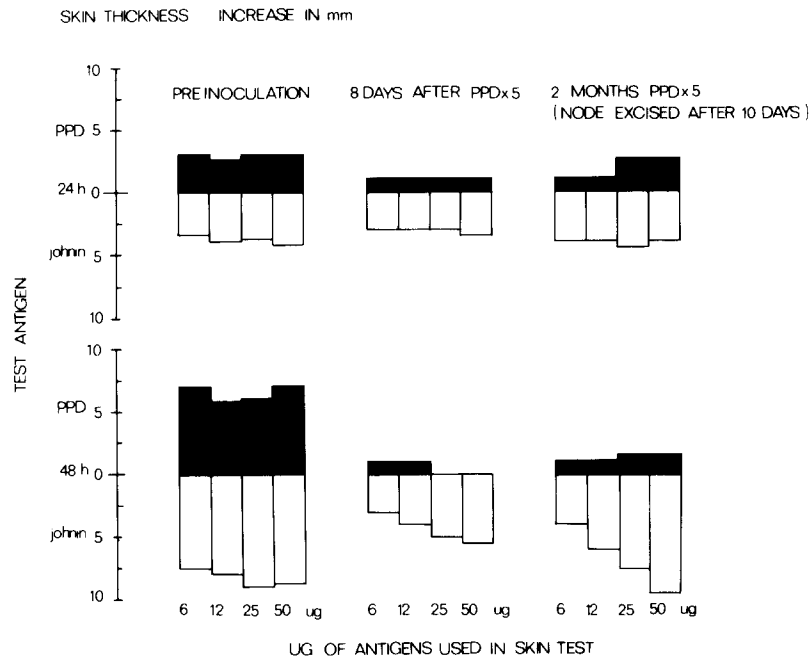


FIG. 2. DTH skin test of sheep DF28. The increase in skin thickness 24 and 48 h after intradermal injections of 100  $\mu$ l of four doses of PPD (black) or johnin (white) is shown.

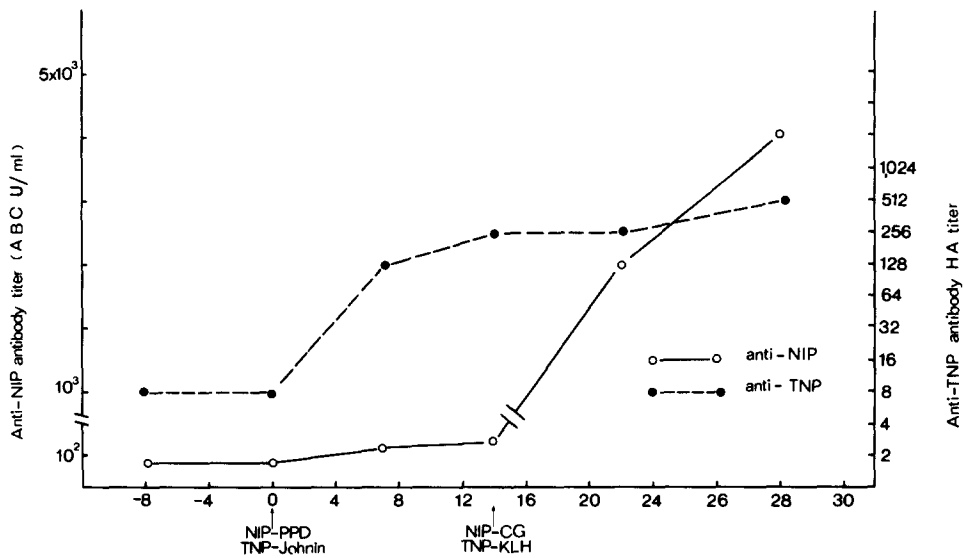


FIG. 3. Anti-hapten antibody response in sheep DF28 after injection of 500  $\mu$ g hapten-carrier conjugates given when sheep was unresponsive to PPD by DTH skin test.

root of the mean (experimental counts per minute - negative control counts per minute). The SD within replicates as  $\pm 15\%$  of the square root of the mean.

*Delayed-Type Hypersensitivity Responses (DTH) Skin Testing.* PPD and johnin were stored at  $-20^{\circ}\text{C}$  at concentrations of 500  $\mu\text{g}$ , 250  $\mu\text{g}$ , 125  $\mu\text{g}$ , and 62.5  $\mu\text{g}/\text{ml}$  in sterile phosphate-buffered

TABLE I  
*Specific Depletion of Systemic Reactivity to PPD in Sheep after Repeated Challenge of a Cannulated Node with PPD*

Sheep	In vitro proliferation of PBL*				DTH skin reaction‡		Serum anti-NIP response§	
	Before		After five PPD injections					
	PPD	MLC or KLH	PPD	MLC or KLH	Before	After five PPD injections	NIP-PPD	NIP-CG
	<i>log<sub>10</sub> cpm</i>				<i>mm</i>		<i>ABC U/ml × 10<sup>2</sup></i>	
7F019	4.51	4.50	2.4	4.59	7.0	0.5	25.0	90
8F002	4.44	4.19	3.1	4.24	8.5	0	10.3	62
8F005	4.5	4.48	1	4.34	6.5	1.5	8.5	45
DF28	4.93	4.59	2.7	4.19	7.0	0	1.25	40
8F024	5.04	4.16	1	4.47	7.0	1.0	3.2	64
513	4.84	4.77	3.72	4.40	8.0	1.0	2.5	18
5F07	4.67	4.83	3.37	4.62	7.0	1.0	4.0	65
6F015	4.76	4.62	3.16	4.60	12.0	0.5	15.0	97
YT9	3.76	3.6	1	3.6	6.0	1.0	5.7	45

\* The in vitro lymphocyte transformation of PBL ( $1.25 \times 10^5$  cells/culture) was measured before the start of the experiment and 5 d after the 5th injection of PPD into the node. Specificity controls were either MLC or KLH. Transformation is measured by [<sup>3</sup>H]thymidine incorporation.

† DTH skin reactions to intradermal injection of 50 µg of PPD both before the start of the experiment and 5 d after the fifth injection of PPD into the node. Before injection, the skin thickness was ~2 mm. The increase in skin thickness to johnin in all sheep after five injections of PPD was always >8 mm.

§ Serum anti-NIP titers after systemic challenge with 500 µg NIP-PPD or NIP-CG given when sheep were skin test negative to PPD. Titers expressed as U of antibody/ml of serum.

|| MLC.

saline (PBS). Skin testing was by intradermal injection of 100 µl of each dose of antigen on the flank of the sheep at sites where the skin thickness before injection did not exceed 3 mm. Thickness of skin was measured by calipers before and at 24 and 48 h after injection. Results are expressed as increase in skin thickness (mm) over the pre-injection measurement.

*Measurement of Anti-Hapten Antibodies.* Anti-NIP antibody was measured by the Farr test (19) using  $10^{-8}$  M 4-hydroxyl-5-[<sup>125</sup>I]iodo-3-nitrophenyl acetyl caproic acid (NIP-CAP) as antigen. NIP-CAP was iodinated using the method of Brownstone et al. (14). [<sup>125</sup>I]sodium iodide was obtained from the Radiochemical Centre. The antigen-binding capacity (ABC) was calculated from the linear position of the binding curve between 3% binding (1 U of antibody/ml) and 30% binding (10 U of antibody/ml). Sera or lymphs were similarly diluted so that at least two dilutions lay between 3 and 30% binding. Anti-TNP antibodies were measured by direct, passive hemagglutination of TNP-coupled sheep erythrocytes (SRBC). The sera were inactivated at 56°C for 30 min before titration.

*Experimental Protocol.* Sheep were primed over a period of several weeks to a variety of antigens. These were bacillus Calmette-Guérin, 10 human doses (Glaxo Laboratories Ltd., Greenford, England); John's disease vaccine, 0.75 ml (*Mycobacterium paratuberculosis*; Central Veterinary Laboratories, Weybridge, Surrey, England); KLH, 1 mg precipitated on alum; NIP-CG, 1 mg on alum; and TNP as TNP-KLH conjugate, 1 mg on alum. All injections were given at different times in several sites intramuscularly. 3 wk after the last injection, the baseline reactivity to PPD and johnin was measured by DTH skin testing. Baseline reactivity of the peripheral blood lymphocytes to PPD, johnin, and KLH (or allogeneic lymphocytes) was assayed by the in vitro proliferation of PBL to these antigens.

The efferent lymphatic draining a single popliteal lymph node was then cannulated and the node challenged by subcutaneous injection of 100 µg of PPD into the drainage area of the node. A further four injections were given at intervals of 4–5 d. The second challenge was

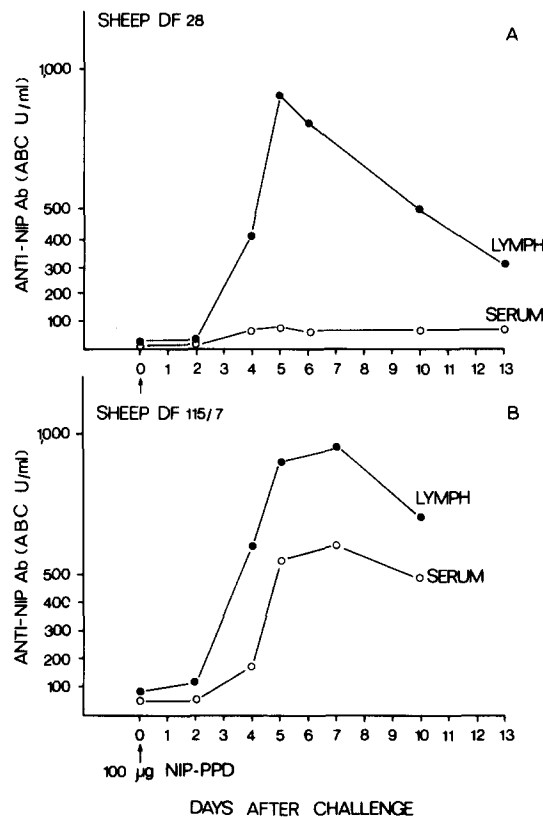


FIG. 4. Anti-NIP antibody titers in efferent lymph plasma and serum of two sheep (DF28 and D115/7) after injection of 100  $\mu$ g NIP-PPD ( $\uparrow$ ) into the drainage area of a cannulated popliteal node. On subsequent postmortem examination the node of D115/7 had a second, noncannulated efferent duct.

always given as NIP-PPD and both the efferent lymph plasma and blood serum later tested for anti-NIP antibody. This was to check that no systemic response occurs after challenge of a cannulated node (see Results). During the entire injection schedule the systemic reactivity of the sheep was monitored by testing the PBL twice weekly for in vitro reactivity to PPD, johnin, and KLH (or allogeneic lymphocytes) by the transformation assay.

After the five injections of PPD the cannulated node was excised and the systemic response to PPD and johnin measured by DTH skin response. Antigen-specific in vivo helper T cell activity was tested by systemic challenge with 500  $\mu$ g NIP-PPD and TNP-johnin. One-half the antigen was given intramuscularly in the shoulder region and the other one-half intravenously. Sera were collected over the next 14 d at which time the sheep were then similarly rechallenged with 500  $\mu$ g NIP-CG and TNP-KLH and later serum samples taken. The anti-NIP antibody titers of the serum samples were measured by the Farr assay and the anti-TNP titers assayed by agglutination of TNP-coupled SRBC. Over the next 2–3 mo, the sheep were tested for reactivity to the antigen by repeated in vitro transformation of PBL and skin testing.

## Results

*Disappearance of Reactivity to Antigen after Local Challenge of Cannulated Lymph Nodes with Antigen.* A total of nine sheep were made systemically unresponsive to PPD after repeated challenge of a cannulated popliteal lymph node with this antigen. Fig. 1 shows the results of the in vitro transformation of the peripheral blood lymphocytes

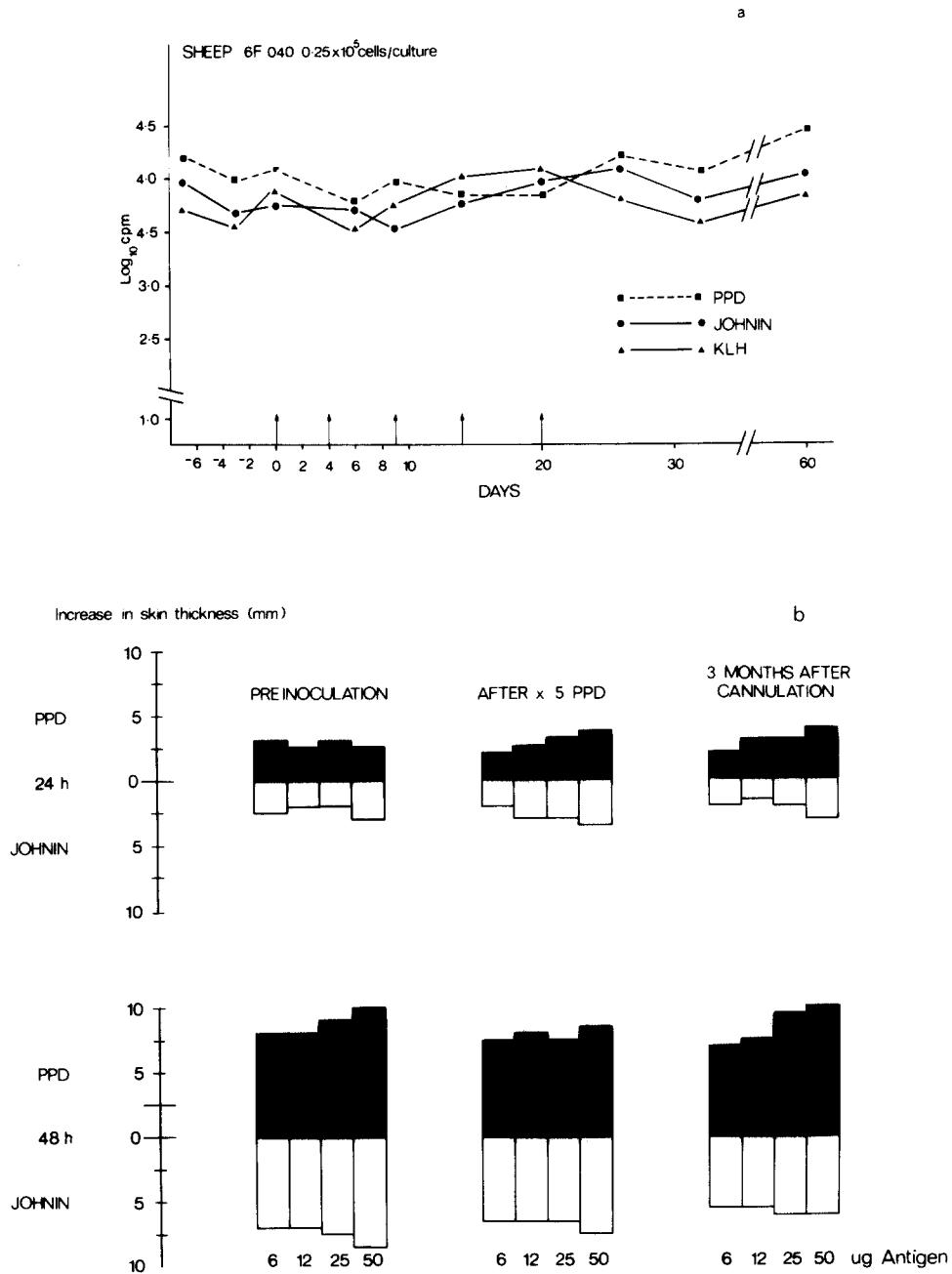


FIG. 5a and b

FIG. 5. Reactivity of sheep 6F040 to various antigens after cannulation of the efferent lymphatic from the right popliteal node and five injections of  $100 \mu\text{g}$  PPD into the drainage area of the uncannulated left popliteal node. (a) In vitro transformation of PBL ( $0.25 \times 10^6$  cells/culture) to PPD, johnin, and KLH. (b) DTH skin response to different concentrations of PPD and johnin. (c) Anti-hapten antibody (Ab) response of sheep 6F040 after systemic challenge with  $500 \mu\text{g}$  hapten-carrier conjugates.



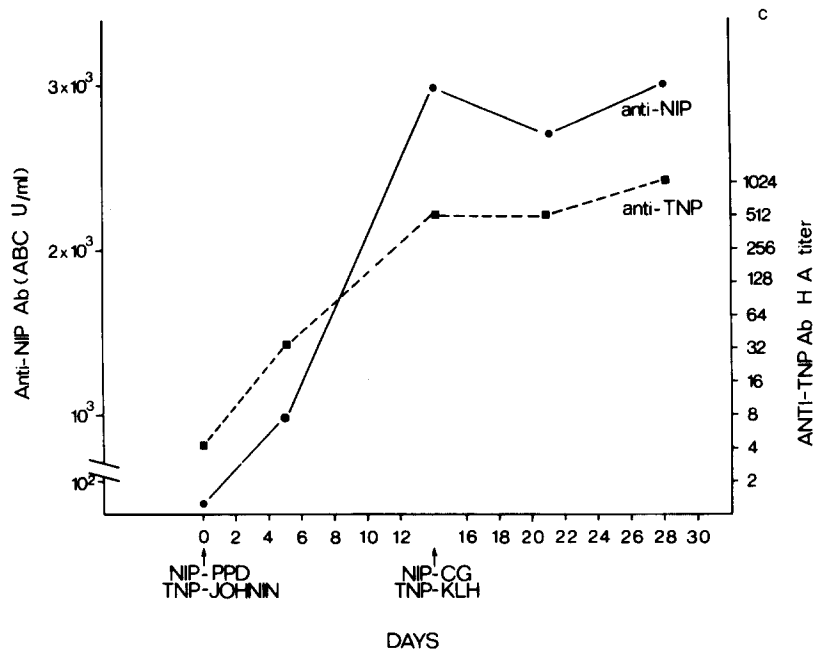


FIG. 5c

of one sheep (DF28) that had been injected five times with PPD. Before cannulation this sheep was equally reactive to all three antigens (PPD, johnin, and KLH) at all four cell concentrations. The reactivity to PPD over the duration of the experiment can be seen to fall. This is most noticeable in the cultures with the lower cell concentrations ( $0.125$  and  $0.25 \times 10^5$ /well) where significant decrease in reactivity is seen after two injections of PPD. At the higher cell concentrations ( $0.625$  and  $1.25 \times 10^5$ /well) this is only obvious after the fourth injection. No significant decrease in the in vitro transformation to johnin and KLH was observed during the same period.

After the fifth injection of PPD the cannulation was terminated and the lymph node excised. The sheep was then skin tested with both PPD and johnin. In contrast to the preinoculation skin test, which showed that the sheep was reactive to both antigens, the skin test after cannulation and five injections of PPD was negative to all four doses of PPD (Fig. 2). Skin test reactivity to johnin was retained.

The absence of systemic T cell reactivity to PPD also applied to T helper cells. Systemic challenge with NIP-PPD produced little serum anti-NIP antibody, whereas challenge 24 h later with TNP on a different carrier (johnin) produced anti-TNP antibody (Fig. 3). This was shown to be a result of loss of PPD-specific T cell and not NIP-specific B cell reactivity, because anti-NIP antibody was produced after systemic challenge with NIP-CG 14 d later. Eight other sheep have been similarly depleted of reactivity to PPD using this procedure; the essential data, after the last injection of antigen when the sheep became unresponsive to the test antigen, are summarized in Table I.

It is known that antigenic challenge of a cannulated node does not result in a systemic response (20). Activated cells and molecules  $>10,000$  mol wt present within the node (21) do not regain the blood at the level of the node. Systemic spread of the

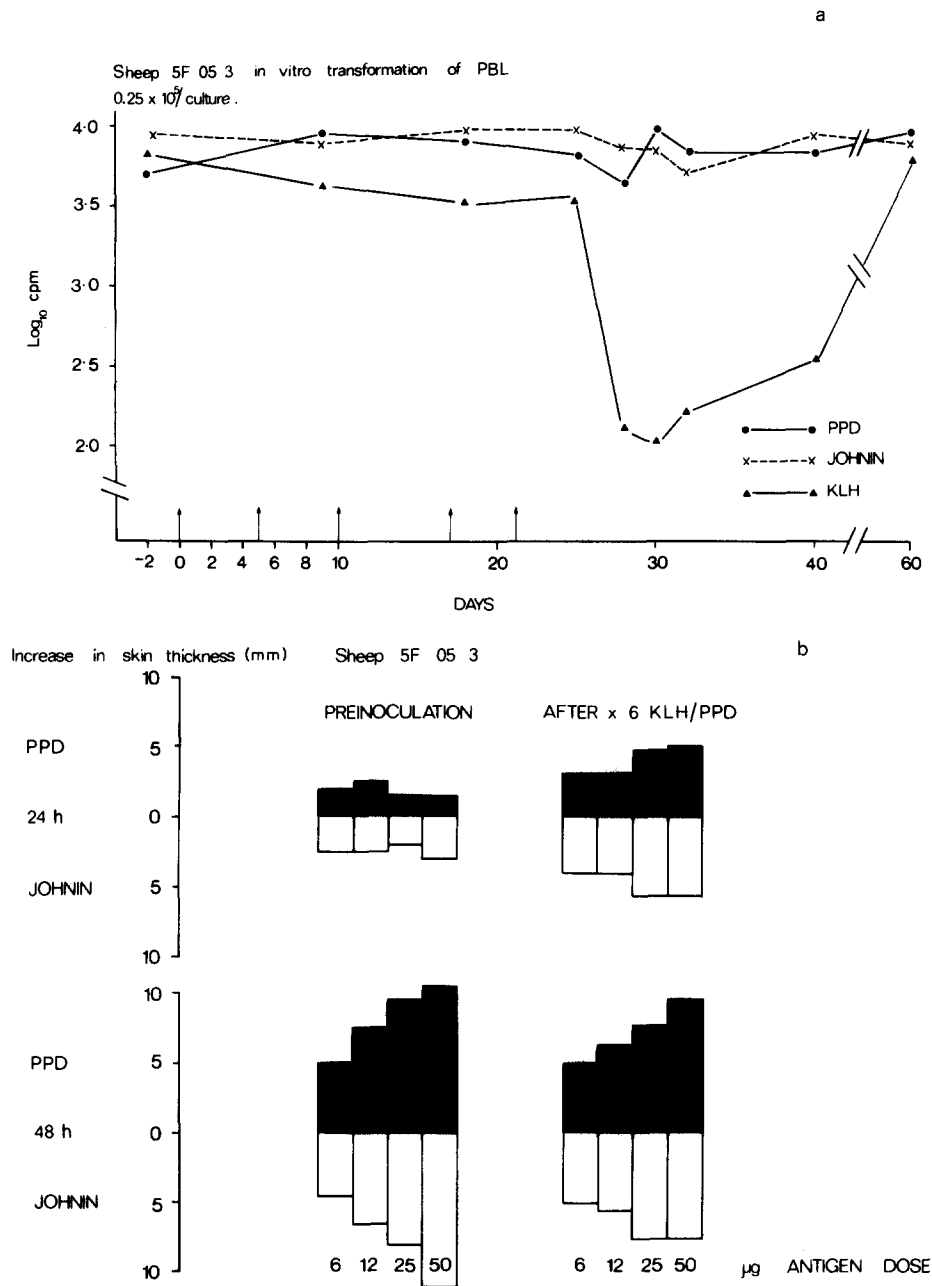


FIG. 6a and b

FIG. 6. Reactivity of sheep 5F053 to various antigens after five injections of  $100 \mu\text{g}$  PPD given simultaneously into the uncannulated left popliteal node. (a) In vitro transformation of PBL ( $0.25 \times 10^5$  cells/culture) to PPD, johnin, and KLH. (b) DTH skin test to different concentrations of PPD and johnin. (c) Anti-hapten antibody response after systemic challenge with  $500 \mu\text{g}$  hapten-carrier conjugates.

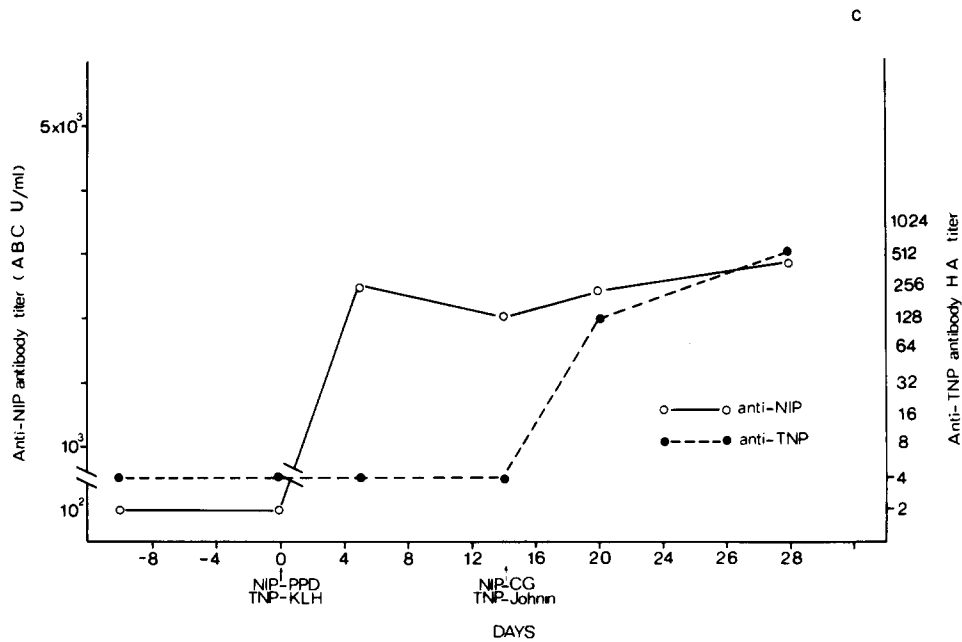


FIG. 6c

response occurs only via the efferent lymphatic and the main lymphatic trunks. The above was confirmed in these experiments. A second challenge of the cannulated node with NIP-PPD produced a brisk anti-NIP antibody response in the efferent lymph without a serum anti-NIP response (Fig. 4a). This shows that PPD-specific helper T cells were present at the beginning of the experiment. When there is escape of antigen or activated cells into the circulation either via collateral lymphatics which bypass the node or because of additional (noncannulated) efferent ducts, then a serum anti-NIP response occurs (Fig. 4b). We have found that when this happens such sheep never become systemically unresponsive even after five injections of PPD.

Attempts to accelerate the rate of depletion by injection of antigen every 24 or 48 h have failed. In these experiments (two sheep) five injections of PPD were given in a 10-d period.

*Failure to Induce Systemic Unresponsiveness after Repeated Challenge of an Uncannulated Node.* We have repeated the experimental protocol in three sheep where the cannulated lymph node was unchallenged and the five injections of PPD given into the uncannulated contralateral node. In these sheep there was no depression of PPD-specific reactivity. The in vitro transformation to PPD (Fig. 5a) remained the same as the antigen specificity controls (johnin and KLH), and there was little diminution of DTH skin response to PPD (Fig. 5b). Furthermore, the level of anti-NIP antibody in the blood serum was the same after injection of 500  $\mu$ g NIP-PPD as after 500  $\mu$ g of NIP-CG (Fig. 5c). This shows that antigen-specific depletion occurs only after prolonged lymphatic drainage of a lymph node undergoing chronic antigenic stimulation and is not just a consequence of the multiple injections of antigen.

In two other sheep these protocols were combined to include internal specificity controls. Five injections of PPD were given into an uncannulated lymph node, while

at the same time KLH was injected into the cannulated node. Figs 6a and 6b show that the PPD-specific response was unaltered whereas *in vitro* transformation of PBL to KLH was depleted. This diminution of KLH reactivity was also shown by the helper T cell assay; no anti-TNP antibody was produced after systemic injection of TNP-KLH (Fig. 6c), although anti-NIP antibody was detected after NIP-PPD challenge. Anti-TNP antibody was produced after TNP-johnin injection.

The *in vitro* transformation to KLH returned to preinoculation levels within 60 d because the challenged lymph node was not excised. We have found that the same applies to sheep depleted of PPD reactivity; failure to excise the challenged node when the animal is systemically unresponsive results in the reappearance of PPD reactivity.

### Discussion

This paper shows that prolonged lymphatic drainage of a lymph node undergoing chronic antigenic stimulation causes systemic unresponsiveness to the challenge antigen. It was shown that repeated challenge of a cannulated node with PPD resulted in gradual loss of PPD-reactivity from the blood as assayed by *in vitro* transformation of peripheral blood lymphocytes. No depletion of reactivity to the other non-cross-reacting antigens (including KLH, johnin, and allogenic lymphocytes) was observed. Total disappearance of PPD reactivity occurred only after the fifth injection of that antigen.

This *in vitro* unresponsiveness was confirmed *in vivo* by the absence of a DTH skin response to PPD and by the inability of the animal to produce an anti-hapten antibody response after systemic challenge with hapten-PPD conjugate. These studies confirm our previous results and further, show that gradual loss of specific reactivity can be monitored during the experiment. Our explanation for this phenomenon is that there is an antigen-specific selective mechanism operating in antigen-stimulated lymph nodes that removes specifically reactive cells from the blood. Random entry of lymphocytes into a stimulated node with total collection of cells and lymph from the cannulated efferent duct cannot account for specific depletion.

We have proposed at least two possible mechanisms for this phenomenon (11, 22). Antigen-specific depletion could be explained by antigen being attached to the luminal surface of the vascular endothelial cells of the PCV. This might preferentially select antigen-reactive lymphocytes into the node. At each passage through the node, only a fraction of the specific cells would be selected from the blood. This would occur in addition to normal random entry. Although no evidence exists for the antigen-specific selection of lymphocytes by endothelial cells at least one example of nonrandom recirculation of lymphocytes is probably determined by direct lymphocyte-endothelium interaction. The preferential migration of intestinal lymph cells through gut-associated lymphoid tissue and peripheral efferent lymphocytes through peripheral lymph nodes (23, 24) seems to be related to the *in vitro* patterns of lymphocyte adherence to the cut surface of the high endothelial venules of lymph node tissue sections (25). Furthermore the finding that endothelia possess membrane Ia antigens (26) may implicate these cells in antigen presentation.

It can be calculated from the kinetics of lymphocyte traffic through lymph nodes that depletion of antigen-reactive cells would occur if there was a two- or three-fold enhancement in the rate of their entry into the stimulated node. Hay and Hobbs (27)

have shown that 60% of the RLP passes through the vascular compartment of a node in the 5 d after antigen stimulation and ~25% of these cells actually enter the node at each passage. From this it follows that 15% of the total recirculating pool is removed by the node every 5 d, leaving 85% of the cells in the circulation.

In our experiments five cycles of antigen stimulation were given over ~25 d. If we ignore the formation of new cells over this period, this would leave  $(0.85)^5$  or 45% of the total RLP. If antigen on the post-capillary venule (PCV) endothelium can increase entry of specific cells by a factor of two (i.e., 30% of the specific cells traffic from blood to lymph), then 70% of the specific cells remain after one cycle. After five such cycles  $(0.7)^5$  or 17% of specific cells will then remain in the RLP. If the extraction rate were three times as efficient, then 55% of the specific cells would remain after one cycle and  $(0.55)^5$  or 5% remain after five cycles. It is at this time that unresponsiveness to PPD is first observed.

The alternative explanation challenges the accepted view that lymphocytes move only from blood to lymph nodes and never in the reverse direction. Entry across the PCV endothelium could be random (28) and all cells which are activated by antigen become retained within the nodes. A proportion of the nonactivated cells returns directly to the blood within the node. Although the work with isolated, perfused rat lymph nodes suggests that retrograde traffic does not occur (29) this need not necessarily apply to nodes undergoing an immune response when marked changes occur in blood flow (27) and lymphocyte traffic through the node (30). Furthermore, recent data (31) strongly suggest that lymphocytes may migrate from lymph nodes to blood in central lymph nodes and that retrograde traffic may be a feature of nodes with a high input of cells from the afferent lymphatic. If specific selection does occur within the node then the likely candidate cells involved are dendritic cells which have been shown to present antigen at the cell surface (32, 33).

It seems unlikely that specific, systemic unresponsiveness induced by the protocol described here is a result of some form of specific desensitization by blocking factors (34) or tolerance induction (35) mediated by suppressor T cells (36, 37). This would require antigen or antigen-specific cells to enter the systemic circulation which does not occur when the efferent lymphatic is cannulated. No anti-hapten response is seen in the serum when antigen is given into the lymph node. Furthermore multiple injections of antigen into an uncannulated lymph node failed to induce unresponsiveness even although a contralateral node was cannulated. We have attempted to test for the presence of a suppressor T cell population in the blood of depleted animals but have failed because of the strong allogeneic differences between individual sheep.

This work is continuing in order to ascertain the actual mechanism of the antigen-specific depletion. We are also attempting to deplete allogeneic responsiveness, which may have some clinical significance.

### Summary

Sheep were primed to a variety of antigens and the efferent lymphatic from a popliteal lymph node was cannulated. The cannulated node was challenged repeatedly with PPD and all the lymph and cells removed from the animal. During this time the PBL were monitored for reactivity to all antigens (purified protein derivative of tuberculin [PPD], johnin, and keyhole limpet hemocyanin [KLH]) by the *in vitro* transformation assay. The response of these cells to PPD was found to be gradually

eliminated after repeated challenge of the cannulated node with that antigen. The response to the other antigens was unimpaired. No depletion of this response to PPD occurred in cannulated sheep when the antigen was given into a noncannulated node. In vivo delayed-type hypersensitivity skin test and helper T cell assays confirmed that the PPD response was specifically eliminated. Our explanation for this result is that there is a specific selection of antigen-reactive cells from the recirculating lymphocyte pool into antigen-stimulated lymph nodes.

We thank Mr. Joher Raniwalla for his excellent technical assistance.

*Received for publication 27 October 1980.*

### References

1. Ford, W. L. 1975. Lymphocyte migration and immune response. *Prog. Allergy*. **19**:1.
2. Ford, W. L. 1968. Duration of the inductive effect of sheep erythrocytes on the recruitment of lymphocytes in the rat. *Immunology*. **15**:609.
3. Rowley, D. A., J. L. Gowans, R. C. Atkins, W. L. Ford, and M. E. Smith. 1972. The specific selection of recirculating lymphocytes by antigen in normal and preimmunized rats. *J. Exp. Med.* **136**:499.
4. Sprent, J., and J. F. A. P. Miller 1973. Effect of recent antigen priming on adoptive immune responses. I. specific unresponsiveness of cells from lymphoid organs of mice primed with heterologous erythrocytes. *J. Exp. Med.* **138**:143.
5. Sprent, J., J. F. A. P. Miller, and G. F. Mitchell. 1971. Antigen-induced selective recruitment of circulating lymphocytes. *Cell. Immunol.* **2**:171.
6. Ford, W. L., and R. C. Atkins. 1971. Specific unresponsiveness of recirculating lymphocytes after exposure to histocompatibility antigen in F1 hybrid rats. *Nat. New Biol.* **234**:178.
7. Atkins, R. C., and W. L. Ford. 1975. Early cellular events in a systemic graft-vs.-host reaction. I. The migration of responding and nonresponding donor lymphocytes. *J. Exp. Med.* **141**:664.
8. Thursh, D. R., and E. E. Emeson. 1972. The immunologically specific retention of long-lived lymphocytes in lymph nodes stimulated by xenogeneic erythrocytes. *J. Exp. Med.* **135**:754.
9. Emeson, E. E. 1978. Migratory behavior of lymphocytes with specific reactivity to alloantigens. II. Selective recruitment to lymphoid cell allografts and their draining lymph nodes. *J. Exp. Med.* **147**:13.
10. Ponzio, N. M., J. M. Chapman-Alexander, and G. J. Thorbecke. 1977. Transfer of memory cells into antigen-pretreated hosts. I. Functional detection of migration sites for antigen-specific B cells. *Cell. Immunol.* **34**:79.
11. McConnell, I., P. J. Lachmann, and M. J. Hobart. 1974. The restoration of specific immunological virginity. *Nature (Lond.)*. **250**:113.
12. Cahill, R. N. P., J. B. Hay, H. Frost, and Z. Trnka. 1974. Changes in lymphocyte circulation after administration of antigen. *Haematologia*. **8**:321.
13. Hall, J. G., and B. Morris. 1962. The output of cells in lymph from the popliteal node of sheep. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **47**:360.
14. Brownstone, A., N. A. Mitchison, and R. Pitt-Rivers. 1966. Chemical and serological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology*. **10**:465.
15. Brownstone, A., N. A. Mitchison, and R. Pitt-Rivers. 1966. Biological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology*. **10**:481.

16. Rittenberg, M. B., and A. A. Amkraut. 1966. Immunogenicity of trinitrophenyl-hemocyanin: production of primary and secondary anti-hapten precipitins. *J. Immunol.* **97**:421.
17. Rittenberg, M. B., K. L. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575.
18. Bøyum A. 1968. Separation of leucocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21 (Suppl)**:97.
19. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I\* BSA and antibody. *J. Infect. Dis.* **103**:239.
20. Hall, J. G., and B. Morris. 1963. Lymph-borne cells of the immune response. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **48**:235.
21. Yoffey, J. M., and F. C. Courtice. 1970. Lymphatics, Lymph and the Lymphomycloid Complex. Academic Press, Inc., New York. 1.
22. McConnell, I., J. Hopkins, and P. J. Lachmann. 1980. Lymphocyte traffic through lymph nodes draining cell shutdown. *Ciba Found. Symp.* **71**:167.
23. Scollay, R. G., J. Hopkins, and J. G. Hall. 1976. Possible role of surface Ig in non-random recirculation of small lymphocytes. *Nature (Lond.)*. **260**:528.
24. Cahill, R. N. P., D. C. Poskitt, H. Frost, and Z. Trnka. 1977. Two distinct pools of recirculating T lymphocytes: migratory characteristics of nodal and intestinal T lymphocytes. *J. Exp. Med.* **145**:420.
25. Butcher, E. C., R. G. Scollay, and I. L. Weissman. 1980. Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. *Eur. J. Immunol.* **10**:556.
26. Hirschberg, H., T. Moen, and E. Thorsby. 1979. Specific destruction of human endothelial cell monolayers by anti-DR antisera. *Transplantation* **28**:116.
27. Hay, J. B., and B. B. Hobbs. 1977. The flow of blood to lymph nodes and its relation to lymphocyte traffic and the immune response. *J. Exp. Med.* **145**:31.
28. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. Lond. B. Biol. Sci.* **159**:257.
29. Sedgley, M., and W. L. Ford. 1976. The migration of lymphocytes across specialized vascular endothelium. I. The entry of lymphocytes into the isolated mesenteric lymph node of the rat. *Cell Tissue Kinet.* **9**:231.
30. Hall, J. G., and B. Morris. 1965. The immediate effect of antigens on the cell output of a lymph node. *Br. J. Exp. Pathol.* **46**:450.
31. Fahy, V. A., H. A. Gerber, B. Morris, W. Travella, and C. F. Zukoski. 1980. The function of lymphocytes in the formulation of lymph. *Monogr. Allergy.* **16**:82.
32. Nossal, G. J. V., G. L. Ada, and C. M. Austin. 1964. Antigens in immunity. IV. Cellular localization of <sup>125</sup>I and <sup>131</sup>I-labeled flagella in lymph nodes. *Aust. J. Exp. Biol. Med. Sci.* **42**:311.
33. Mitchell, J., and A. Abbott. 1965. Ultrastructure of the antigen-retaining reticulum of lymph node follicles as shown by high-resolution autoradiography. *Nature (Lond.)*. **208**:500.
34. Brent, L. 1972. Tolerance and enhancement in organ transplantation. *Transplant. Proc.* **4**:363.
35. Uhr, J. W., and A. M. Pappenheimer. 1958. Delayed hypersensitivity. III. Specific desensitization of guinea pigs sensitized by protein antigens. *J. Exp. Med.* **108**:891.
36. McCullagh, P. 1975. The mechanism of unresponsiveness in lymphocytes specifically selected by antigen in normal rats. *Aust. J. Exp. Biol. Med. Sci.* **53**:175.
37. McCullagh, P. 1980. Unresponsiveness of recirculating lymphocytes after antigenic challenge. *Monogr. Allergy.* **16**:143.